TRANSLATION SYSTEM

The present invention relates to a cell-free translation system. In particular, the invention relates to a cell-free translation system derived from cell extracts, that reproduces the synergism that occurs during translation *in vivo* between the *cap* and the *poly(A)* tail structures of the mRNA.

Translational control is an important mechanism for the regulation of gene expression during a variety of biological processes, including cellular metabolism (Hentze & Kuhn, (1996) P.N.A.S. USA 93: 8175-8182), cell differentiation (Ostareck *et al.*, 1997, Cell. 89: 597-606) and embryonic development (Wickens *et al.*, 1996, in Translation Control, Eds. Hershey, J.W.B., Mathews, M.B. and Sonenberg, N. (Cold Spring Harbour Lab. Press, Plainview, NY, pp. 411-450). Failure to regulate translation properly often leads to disease.

In vivo, two modifications in the mRNA, namely a *cap* structure at the 5' end and a *poly(A)* tail at the 3' end, act synergistically to promote mRNA translation (Gallie, 1991, Genes Dev. 5: 2108-2116). To date, research into the function of those structures and the synergism that occurs between them has been restricted by the lack of *in vitro* systems that accurately recapitulate these features.

Recently, a cell-free translation system that reproduces this synergism has been obtained from the unicellular eukaryote *Saccharomyces cerevisiae* (Iizuka *et al.*, 1994, Mol. Cell. Biol. 17: 7322-7330). Capped mRNAs were found to be translated 50-fold more efficiently than uncapped mRNAs in yeast lysate. Polyadenylated RNAs were found to be translated 140-fold more efficiently than non-polyadenylated RNAs. mRNAs containing both a 5' *cap* and a 3' *poly(A)* tail were found to be translated several hundred-fold more efficiently than mRNAs lacking both of these terminal sequences.

A cell-free translation system derived from animal cells that is capable of reproducing this synergism would be of great value for a number of reasons. Firstly, this would facilitate and enhance the validity of basic research into the factors and mechanisms involved in translational control in higher eukaryotes, including the regulation of translation by cis- and trans- acting factors, the molecular basis of synergism, mRNA

stability and polyadenylation. Second, species-specific differences in translational mechanisms could be evaluated. Such a system would also allow the improvement of large-scale protein synthesis, both with respect to the efficiency of translation and to the fidelity of post-translational modification.

Summary of the Invention

According to a first aspect of the present invention, there is provided a method for the *in vitro* translation of a ribonucleic acid template, said ribonucleic acid having both a 5' cap and a 3' poly A tail, said method comprising incubating a cell extract of a multicellular eukaryote with said ribonucleic acid template under conditions such that translation of the RNA template to produce its encoded protein by one or more components in the cell extract occurs and the amount of the encoded protein thus produced is greater than the total of (a) the amount of the encoded protein that is produced under said conditions when the ribonucleic acid template has a 5' cap but no 3' poly A tail, plus (b) the amount of the encoded protein that is produced under said conditions when the ribonucleic acid template has a 3' poly A tail but no 5' cap.

It has been found, surprisingly, that by combining a dechorionation procedure that has been previously used for the study of chromatin assembly (Becker *et al.*, 1994) with homogenisation and centrifugation conditions used to study translation (Scott *et al.*, 1979) a protocol results that allows the preparation of cell extract with greatly improved properties over conventionally known extracts for use in *in vitro* translation procedures.

The cell extract may be an animal cell extract, such as a mammalian cell, or an insect cell. Specific examples include cell extracts of Drosophila, C. elegans, rodent, rabbit (for example, reticulocyte cells), human (for example HeLa cells) and primate cells. The cells may be embryonic or adult or from a particular tissue such as the ovaries. Preferably, the cell extract is a Drosophila cell extract, most preferably a Drosophila embryo or ovary cell extract.

The extract exhibits properties that were previously unforeseen, such as the capability to reproduce the synergism that occurs during translation *in vivo* between the cap and the poly(A) tail structures of an mRNA molecule, and the capability to perform mRNA polyadenylation. In addition, the procedure is simple and allows the preparation of large

amounts of extract at low cost. This makes the system useful in both basic research and in commercial applications as is discussed in more detail below.

RNA templates suitable for use in the methods of *in vitro* translation of the invention may be recombinant or can be purified native templates. In preferred aspects of the invention, the poly(A) tail of the RNA template can be encoded in a DNA coding sequence which can be transcribed to generate an RNA template with a poly(A) tail of defined length. An alternative method of generating the poly(A) tail is the use of a poly(A) polymerase in an *in vitro* reaction to add the tail to the template as a post-transcriptional modification.

The 5' cap portion may be added co-transcriptionally to the RNA template by the RNA polymerase. A suitable protocol can be found in "Protocol and Applications Guide"; Promega, 2nd edition, p62. If the template is a purified native RNA template, the cap structure will already be in place.

According to a second aspect of the invention there is provided a method for the preparation of a Drosophila embryo extract comprising the steps of a) dechorionating Drosophila embryos in an aqueous isotonic buffer comprising detergent and bleach; b) washing the embryos; c) homogenizing the embryos to produce a homogenate; d) centrifuging the homogenate; and e) recovering non-pelleted material from the centrifuged homogenate. This cell extract is particularly suited for use in the method of the first aspect of the invention.

The age of the embryo is not crucial to the method of the invention, although embryos of between the ages of 1 hour and 12 hours of development are preferred.

Before preparation of extract, the embryos should preferably be collected in a sieve and washed extensively with water to remove debris from the culture medium in which they have been laid. For preparation of extract, the embryos are washed with an aqueous buffer, preferably with fresh EW buffer (0.7% NaCl, 0.04% Triton X-100), and poured into a suitable vessel. An excess of buffer is then added and the embryos allowed to settle. At this stage, floating embryos should be discarded. For dechorionation, fresh buffer should be added and the embryo suspension agitated, preferably using a spinning

magnet or other suitable device. In all steps for the preparation of cell extract, ethanol-based buffers should be avoided.

For dechorionation, bleach is added to the embryo suspension, preferably at a concentration of about 3% by volume and the solution incubated for an appropriate time. Preferably, incubation is for between 1 and 9 minutes, most preferably 3 minutes.

Dechorionation may be carried out at a temperature of between 18 and 37°C. Preferably, dechorionation is carried out at about 25°C.

After dechorionation, embryos are preferably transferred to a sieve and washed extensively with water. Dechorionated embryos are then transferred to a suitable vessel (such as a beaker or cylinder) and buffer such as DE buffer (10mM HEPES pH 7.4, 5mM DTT) added. Floating embryos are discarded. Preferably, about 1 volume (with respect to the volume of settled embryos) of fresh buffer containing protease inhibitors is added to prevent proteolysis of components of the extract.

Embryos are then homogenised. Preferably, homogenisation is carried out at about 4°C. Most preferably, a Potter-Elvehjem homogeniser is used at about 1500 rpm (about 20 strokes). The homogenate should preferably be kept on ice to minimise proteolysis.

The homogenate is preferably centrifuged, ideally in an ultracentrifuge at 24,000 rpm (40,000g)/TLS-55 rotor/4°C/20 minutes. The clear interphase is taken, ideally by puncturing the tube with a syringe, and is transferred to a Falcon tube.

Glycerol may then be added to the extract, preferably to a 10% final concentration, and the extract aliquoted, frozen in liquid nitrogen and kept at -70°C for future use.

Drosophila ovaries may also be used to produce a cell extract. The procedure is as follows: adult female flies are manually dissected to obtain the ovaries, which should be placed in an eppendorf tube on ice in an aqueous medium, for example PBS. Ovaries are then allowed to settle, before transferring the ovaries slowly from an isotonic medium to a hypotonic medium. For example, the ovaries can be washed twice with around 12 volumes of a 1:1 mix of PBS:DEI buffer (DEI = 10mM HEPES pH 7.4, 5mM DTT, 1 x COMPLETE-Protease inhibitors from Boehringer Mannheim cat#1697498), and twice with 12 volumes of DEI. After washing, all the buffer is

removed and the ovaries manually homogenised using a plastic pestle. The homogenate can then be spun and the extract collected as described above for embryo extract.

The invention also provides a method for the preparation of a mammalian cell extract comprising the steps of a) collecting cells by centrifugation; b) washing the cells; c) resuspending the cells; d) homogenising the cells to produce a homogenate; e) centrifuging the homogenate and f) recovering non-pelleted material from the centrifuged homogenate. This cell extract is well-suited to use in the method of the first aspect of the invention.

Before preparation of extract, the cells should be grown in culture, preferably at exponential growth. Suspension cultures grown in, for example Jocklik's medium supplemented with 5% newborn bovine serum at 37°C, are suitable. The cells may be collected from culture by centrifugation, for example, by harvesting at 700g for 15 minutes. For preparation of extract, the cells should be washed with phosphate buffered saline at 4°C, before resuspension in a suitable ice cold hypotonic buffer such as a buffer containing containing Hepes 10 mM. PH 7.6. KOAc 10 mM. $Mg(OAc)_2$ 0.5 mM and Dithiothreitol 5 mM. Reduced amounts of DTT are not recommended and addition of protease inhibitors is optional.

After a short period on ice, for between 2 and 10 minutes, the cells are homogenised. Preferably, homogenisation is carried out at about 4°C. Most preferably, the cells are homogenised using between 10 and 30 strokes of a Dounce homogeniser (pestle type B). The homogenate should preferably be kept on ice to avoid proteolysis.

The homogenate is then centrifuged, ideally at 14000g for 5 minutes at 4°C. A longer centrifugation is not recommended. Supernatant fluid is divided into aliquots frozen in liquid nitrogen and stored at -80° C. Addition of glycerol is not necessary.

According to a third aspect of the invention there is provided the extract of Drosophila embryos or ovaries, or of mammalian cells, produced according to the methods of the second aspect of the invention.

According to a fourth aspect of the invention there is provided a method for the *in vitro* translation of a ribonucleic acid template, comprising the steps of adding a ribonucleic

acid template to a translation mix in the presence of the embryo, ovary or mammalian cell extract of the third aspect of the invention to form a reaction mix and incubating the reaction mix for at least 90 minutes at between 18°C and 28°C. Preferably, the reaction mix is incubated for at least 90 minutes at 25°C.

By reaction mix is meant a solution of components necessary for *in vitro* translation to occur. Such components may include any of the following: spermidine, amino acids, creatine phosphate, creatine kinase, dithiothreitol (DTT), buffer, $Mg(OAc)_2$, KOAc, tRNA, cell extract and RNA template. This reaction mix should preferably be prepared fresh. All the components of the reaction mix, except the creatine kinase, should be dissolved in distilled water to their required concentrations.

Preferably, the buffer is HEPES buffer, although any buffer with a low concentration of salt (below 10mM) may be used. HEPES buffer is most preferably used at a final concentration of between 16 and 24mM. As used herein, the term "final concentration" means the concentration of the component that is present in the *in vitro* translation mix. These concentrations are optimal for the translation reaction to take place.

Preferably, said tRNA is calf liver tRNA, at a final concentration of below 150µg/ml, most preferably about 100µg/ml.

For Drosophila ovary or embryo extract, the final concentration of spermidine is preferably about 0.1mM. For amino acids, final concentration is about 60µM. For creatine phosphate, the concentration can be in the range 17-23mM. It is shown herein (see Figure 6) that it is preferable to use fresh or newly thawed creatine phosphate. The level of translation doubles when fresh creatine phosphate is used. Creatine kinase should be used at a concentration of about 0.08mg/ml.

The concentrations of DTT, $Mg(OAc)_2$ and KOAc should be optimised for each individual mRNA template, as will be appreciated by those skilled in the art. For example, for translation of M1414WT (bgal) (see below), ideal concentrations are DTT, 1.2mM; $Mg(OAc)_2$, 0.6mM; and KOAc, 60mM. For c-Luc-a, ideal concentrations are DTT, 0mM; $Mg(OAc)_2$, 0.4mM; and KOAc, 30mM.

However, final concentration of $Mg(OAc)_2$ can be in the range 0-3mM. KOAc can range between 0 and 200mM. DTT can range between 0-4mM.

Embryo or ovary extract should preferably be used at a concentration of about 40% by volume.

For mammalian cell extract, the final concentration of spermidine is preferably about 0.1mM. For amino acids, final concentration is about 100μM. For creatine phosphate, the concentration can be in the range 17-23mM. Creatine kinase should be used at a concentration of about 0.05mg/ml. As stated above for Drosophila extract, the concentrations of $Mg(OAc)_2$ and KOAc should be optimised for each individual mRNA template, as will be appreciated by those skilled in the art. For example, suitable concentrations may be along the following lines; $Mg(OAc)_2$, 2.5mM; KOAc, 50mM.

However, final concentration of $Mg(OAc)_2$ can be in the range 0-3mM. KOAc can range between 0 and 200mM. DTT can range between 0-4mM.

Mammalian cell extract should preferably be used at a concentration of about 40% by volume.

Optimal RNA template concentration will vary depending upon the RNA type. The skilled artisan will appreciate that optimisation of the system may be easily performed for a particular RNA species of choice using routine procedures. For example, for the species c-luc-a (coding for firefly luciferase) a concentration of about 3.2ng/μl is optimal using Drosophila embryo extract. The mRNA template should preferably be capped and should contain a *poly(A)* tail of more than 31 adenine nucleotides (more preferably 70-100 adenines).

For each mRNA species, preliminary reactions should be performed so as to obtain a curve of translation efficiency with differing amounts of mRNA. An amount of mRNA should be used in the linear range of translation. In the examples described herein, test reactions were performed with M1414WT (bgal) [this codes for the β-galactosidase enzyme and contains 5' and 3' UTR sequences from Drosophila oskar mRNA], c-Luc-a and Luc mRNA (Promega).

Reaction mix prepared in this fashion contributes to the advantageous features of the method of this aspect of the invention, namely reproducing the synergy between the 5' *cap* and 3' *poly(A)* tail of the RNA molecule for translation.

Additionally to this synergy, the level of translation is enhanced, so leading to a greater production of protein. The fidelity of post-translational modification appears also to be retained, differentiating the system of the present invention over that described previously in yeast (Iizuka *et al.*, 1994). Although yeasts do possess enzymes that are capable of effecting the post-translational modification of proteins, the type and extent of modification tends to differ from that found in higher eukaryotes. Consequently, the Drosophila system of the present invention generates proteins modified similarly to the state in which they are found *in vivo*.

The incubation step is preferably performed for sufficient time to allow the translation reaction to proceed to the extent desired. Preferably, the incubation step is performed for at least about 90 minutes.

Ranges of suitable concentrations of components of the reaction mix for Drosophila extract, along with optimal concentrations, are given below in Table 1. These concentrations were optimised for luc and c-luc-a mRNA species, coding for the firefly luciferase enzyme.

TABLE 1

	Range tested	**Optimal**	**RNA**	**Observations**
Creatine phosphate	0-168mM	17-23mM	Luc	Use fresh (thaw only once)
Creatine kinase	0-1.68 mg/ml	0.08 mg/ml	Luc	
KOAc	0-200mM	74mM	Luc	Should be optimised for each RNA template
$Mg(OAc)_2$	0-3mM	0.3mM	Luc	Should be optimised for each RNA template

Spermidine	0-1.8mM	0.1mM	c-Luc-a	
Amino acids	0-100 µM	60 µM	c-Luc-a	
DTT	0-4mM	0mM	c-Luc-a	Should be optimised for each RNA template
tRNA	0-150 µg/ml	100 µg/ml	Luc	
Temperature	18-37°C	25°C	c-Luc-a	

Ideally, the *in vitro* translation reaction is performed in a final volume of about 12.5 microlitres. Below, in Table 2, the volume of stock concentrations is given in order to make up a reaction volume of 12.5 microlitres.

TABLE 2

- Final volume = 12.5 µl
- Mix per reaction:

	Volume (µl)	Final conc.
2.5mM spermidine	0.5	0.1mM
1mM amino acids (Promega)	0.75	60 µM
1 M creatine phosphate (fresh or newly thawed)	0.21	16.8mM
10 µg/µl creatine kinase	0.1	80 ng/µl
0.1 M DTT	0.15	1.2mM
1 M HEPES pH 7.4	0.3	24mM
4.13mM $Mg(OAc)_2$	1.8	0.6mM

1 M KOAc	0.75	60mM
1.1 µg/µl calf liver tRNA	1.1	100ng/µl
Embryo extract	5	40%
RNA template	1	3.2ng/µl
H_2O	to 12.5 µl final	

For HeLa cell extract, the reaction mix may be as follows:

Untreated extract	40% of the final volume
Hepes buffer, pH 7.6	16 mM
KOAc	50 mM
$Mg(Oac)_2$	2.5 mM
amino acid mixture	100 µM
spermidine	0.1 mM
ATP	0.8 mM
GTP	0.1 mM
Creatine phosphate	20 µM
Creatine kinase	50 ng/µl
Calf liver tRNA	100 ng/µl
RNA template	1 ng/µl

These optimal values were obtained for this translation assay by testing a range of components concentrations with a capped and polyadenylated mRNA expressing the bacterial enzyme chloramphenicol acetyltransferase (CAT). The amount of the CAT

protein produced was measured using a colorimetric enzyme immunoassay (CAT ELISA, Böhringer Mannheim).

According to a fifth aspect of the invention there is provided the use of cell extract according to the third aspect of the invention in a method of *in vitro* translation of a ribonucleic acid template, such as the methods of the first and fourth aspects of the invention. The method reproduces the synergism exhibited *in vivo* between the 5' *cap* and 3' *poly(A)* tail of the ribonucleic acid template.

According to a sixth aspect of the invention there is provided the use of cell extract according to the third aspect of the invention or method of first or the fourth aspects of the invention to screen for compounds that either a) decrease the translation, polyadenylation and/or stability of all mRNA species, or of specific mRNAs; b) potentiate the translation, polyadenylation and/or stability of all mRNA species, or of specific mRNAs; or c) decrease or potentiate the synergism between the 5' cap and the 3' poly(A) tail of the mRNA. Suitable compounds may interact (either directly or indirectly) with only one of the 5' cap or poly(A) structures. Preferably, compounds identified in such a screen have an effect on translational control in higher eukaryotes, in particular insects. This screening method has advantages over prior art methods because the synergy exhibited between cap and poly(A) structures mimics the features of *in vivo* translation and allows the screening in an *in vitro* system for modifiers effecting translation that are significantly more likely to function *in vivo*.

According to a seventh aspect of the invention there is provided a compound identified by a screen according to the sixth aspect of the invention. Suitable compounds may be natural or synthetic, for example, an aptamer, or a peptide and may be useful for therapy of diseases and for the control of plagues and diseases caused by insects, for example malaria and plant pestilence. For example, the compound may that interact with 5' *cap* or 3' *poly(A)* structures of an mRNA template or affect in some way the synergism between the 5' *cap* and 3' poly (A) tail of an mRNA template. In some instances, compounds may be specific for a certain group of mRNA templates, or for mRNA templates within a certain species. It is envisaged that such compounds will prove useful in the control of plagues and diseases caused by insects, for example malaria and plant pestilence, by interfering with the expression of certain essential insect proteins.

According to an eighth aspect of the invention there is provided the use of the cell extract according to the third aspect of the invention or method of the first or fourth aspects of the invention in research into the regulation and function of post-transcriptional mRNA modification or post-translational protein modification.

After the identification of the polypeptides that are responsible for the synergism between the 5' cap and the 3' poly(A) structures of the mRNA, it is possible to study the productive interactions between them and to define the domains and amino acid residues that are responsible for these interactions. It will, then, become possible to design chimeric molecules that comprise the effector domains from these polypeptides fused to other polypeptides of interest. Upon interaction of the polypeptides of interest, the effector domains would promote the translation of a reporter mRNA, such as luciferase. The level or degree of interaction would be quantified by measuring the amount of light that is produced. In order to aid the study of interactions of interest, appropriate components such as Drosophila embryo extract or HeLa cell extract, reporter mRNA and reaction mix may be provided in the form of a kit.

According to a ninth aspect of the invention there is provided a kit for the analysis of *in vitro* translation, polyadenylation and/or stability of ribonucleic acid. The kit should contain cell extract according to the third aspect of the invention along with stock solutions of all the other components of the translation mix in appropriate quantities. The kit will therefore in addition comprise any one or all of spermidine, amino acids, creatine phosphate, creatine kinase, optionally dithiothreitol (DTT), buffer, $Mg(OAc)_2$, KOAc, tRNA, and RNA template.

As noted above, preferably, the buffer is HEPES buffer, the tRNA is calf liver tRNA and ideally, the creatine phosphate is made fresh. However, creatine phosphate may be supplied in powdered form in the kit for dissolution in distilled water as and when required for use.

Generally, an RNA template will not be supplied as part of the kit, since in the main part, it is envisaged that the kit will be purchased for the purposes of experimental research. However, a control RNA template may be included in the kit so that a user can ensure that the translation reaction is proceeding appropriately. For example,

comparison with a control RNA template may be used to optimise the concentration of test RNA, DTT, $Mg(OAc)_2$ and KOAc that is to be used.

The kit preferably also contains appropriate instructions to enable a user to perform the *in vitro* translation reaction appropriately.

The invention will now be described in detail with particular reference to a Luc mRNA template. As will be clear to the person of skill in the art, variations from the described protocol may be made without departing from the scope of the invention.

All references cited herein are hereby incorporated by reference in their entireties.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows comparative results of translation levels and levels of synergy found between 5' *cap* and 3' *poly(A)* tail of an RNA template.

Figure 2 is an expanded scale of the results of Figure 1.

Figure 3 shows optimisation of Mg^{2+} concentration.

Figure 4 shows optimisation of K^+ concentration.

Figure 5 shows optimisation of creatine kinase concentration.

Figure 6 shows how percentage translation is improved by using fresh creatine phosphate.

Figure 7 shows optimisation of creatine phosphate concentration.

Figure 8 shows optimisation of spermidine concentration.

Figure 9 shows optimisation of tRNA concentration.

Figure 10 shows optimisation of temperature, amino acid concentration and DTT concentration using c-luc-a RNA.

Figure 11 shows the time course of translation of CAT mRNAs using the HeLa cell extract.

Figure 12 shows Northern blot analysis of the transcripts during the translation assay. Bars represent radioactive intensity of each messenger measured by a phosphoimager. Graph (A) shows the four messengers at different times, from 0 to 150 minutes. Graph (B) shows the Cap and the Cap-pA transcripts from 0 to 90 min.

Figure 13 shows time course of translation of the Luc messengers. Efficiency of translation is represented by light emission measured by a luminometer. Graph (B) shows the data represented in Graph (A) with the y axis maximum at 20000 luminescence instead of 550000.

EXAMPLES

Example 1: Preparation of extracts from Drosophila embryos

Extracts from 1h30', 3h, 6h and 12h embryos were prepared with similar results.

Embryos were laid by 2-3 day old adult Drosophila flies on agar-apple juice plates (2.9% Agar; 30% Apple Juice; 4.4% Rubensirup; 0.25% Nipagin). The plates, spread with some fly food (220ml deionized water, 1.4ml Propionic acid; 150g dry yeast), were left overnight for embryo laying). Embryos from 16 agar-apple juice plates were collected in a pile of sieves (the first with a cut-off size of an adult fly, the second with a cut-off size of a fly appendix [e.g.: a leg, a head or an antenna], the last with a cut-off size of a single embryo) and washed extensively (~5-10 minutes) with tap water to remove debris coming from the plates.

Embryos were subsequently washed with freshly prepared isotonic EW buffer (0.7% NaCl, 0.04% TritonX-100). To do this, embryos were transferred to a 500ml cylinder containing EW buffer, and were allowed to settle for ~3-5 minutes (roughly the time needed for 90% of the embryos to pellet by gravity) and were then washed twice with 500ml EW Buffer. Floating embryos were eliminated by suction.

Embryos were dechorionated in the 500 ml cylinder at room temperature (~20-25°C) with 260ml of EWB (0.7% NaCl, 0.04% TritonX-100, 3% Sodium Hypochlorite) for 3 minutes under vigorous agitation provided by a magnetic stirrer. Sodium Hypochlorite was from Sigma, Thomas Chemikalien, or U.S. CHLOROX. No significant change in the translation efficiency of the extract was noted for different bleach types.

Dechorionated embryos were transferred back to the sieves, and were vigorously and extensively washed with tap water (by flushing a strong stream of water for about 5-10 minutes). Washed embryos were settled by gravity twice with 100 ml of DE buffer (10mM HEPES pH 7.4, 5mM DTT) in a 100ml cylinder. Floating embryos were discarded, and an equivalent of one volume (with respect to the settled embryos) of DEI buffer (10mM HEPES pH 7.4, 5mM DTT, 1x COMPLETE-Protease Inhibitors from Boehringer Mannheim cat# 1697498) was added.

Embryos in DEI buffer were homogenised in a cold room (~4°C) by 20 strokes of a Potter-Elvehjem homogeniser at 1500 rpm and the homogenate was kept on ice. The homogenate was spun in a table-top ultracentrifuge (Beckmann) at 24000 rpm (40000x g) in a TLS-55 rotor at 4 C for 20 minutes. The clear aqueous interphase was taken by puncturing the tube with a syringe, and was transferred to a Falcon tube. Glycerol was added to 10% final, and the extract was aliquoted, flash-frozen in liquid nitrogen and stored at -70°C.

Example 2:

2.1 *In vitro* translation

A typical experiment is shown below.

The reaction is performed in a final volume of 12.5 µl, containing the following mix (a):

	volume (µl)	Final conc.	
2.5 mM spermidine	0.5	0.1 mM	
1mM amino acids	0.75	60 µM	
1 M creatine phosphate (fresh or newly thawed)	0.21	16.8 mM	
10 µg/µl creatine Kinase	0.1	80 ng/µl	
0.1 M DTT	Y	0 - 1.2 mM	(b) (*)
1 M HEPES pH 7.4	0.3	24 mM	
4.13 mM Mg(OAc)2	Z	0.3 - 0.6 mM	(b) (*)
1 M KOAc	W	30 - 80 mM	(b) (*)
1.1 µg/µl calf liver tRNA	1.14	100 ng/µl	

embryo extract	5	40%
RNA template	X	(c)
H_2O	to 12.5 µl final	

The reaction was incubated at 25°C for 90 min.

(a) A master mix should be prepared for as many samples as it is required.

(b) Should be optimised for each mRNA template.

(c) The mRNA template should be capped and should contain a poly(A) tail of more than 31 A's (usually 70-100 A's), although mRNAs without a poly(A) tail are also translated albeit at lower efficiency. A curve of translation with different amounts of RNA should be performed to use an amount in the linear range of translation.

(*) Concentrations (**mM**) optimal for:

RNA	DTT	Mg(OAc)2	KOAc
M1414WT (bgal)	1.2	0.6	60
c-Luc-a	0	0.4	30
mLuc-a	0	0.6	80
c-CAT-a	1.2	0.6	60

M1414WT RNA encodes for the β-galactosidase enzyme (bgal) and contains 5' and 3' UTR sequences from Drosophila oskar mRNA. *c-Luc-a* encodes for the firefly luciferase enzyme. *mLuc-a* encodes for the firefly luciferase enzyme, and contains the 5' UTR of Drosophila msl-2 mRNA. *c-CAT-a* encodes for the chloramphenicol acetyl transferase enzyme. All RNAs are capped and polyadenylated.

Spermidine is from SIGMA, cat# S-0381.

Amino acids were obtained from Promega (cat #L4461), or can be self-made from a SIGMA kit (cat# LAA-21).

Creatine Kinase is from Boehringer Mannheim (cat# 126969). The stock solution is prepared at 10 mg/ml in 50% glycerol, 20 mM HEPES-KOH pH 7,4.

Using this protocol, together with the extract described in Example 1,high levels of translation and synergy can be obtained, compared to other methods (see Figure 1).

2.2 Optimisations

Ranges of suitable concentrations, along with optimal concentrations, are given below.

The method of Example 1 was followed (not that of Scott *et al*), and one concentration value was varied for each experiment. Luc RNA from Promega was used for these experiments, except where otherwise stated.

Figure 3 shows optimisation of Mg^{2+} concentration. Optimum concentration was found to be 0.3mM. Figure 4 shows optimisation of K^{+} concentration. Optimum concentration was found to be 74mM for this system.

Figure 5 shows optimisation of creatine kinase concentration. Optimum concentration was found to be 0.08mg/ml for this system.

Figure 6 shows how % translation could be improved more than two times by using fresh creatine phosphate.

Figure 7 shows optimisation of creatine phosphate concentration. Optimum concentration was found to be 23mM for this system.

Figure 8 shows optimisation of spermidine concentration. Optimum concentration was found to be 0.1mM for the c-luc-a RNA system.

Figure 9 shows optimisation of tRNA concentration. Optimum concentration was found to be 100µg/ml for the luc RNA system.

Figure 10 shows optimisation of temperature, amino acid concentration and DTT concentration using c-luc-a RNA. Optimum temperature was found to be 25°C. Optimum amino acids concentration was found to be 60µM. Optimum DTT concentration was found to be 0mM.

	Range tested	Optimal	RNA	Observations
Creatine phosphate	0 - 168 mM	17 - 23 mM	Luc	Use fresh (thaw only once)
Creatine kinase	0 - 1.68 mg/ml	0.08 mg/ml	Luc	
KOAc	0 - 200 mM	74 mM	Luc	Should be optimised for each RNA template
Mg(OAc)2	0 - 3 mM	0.3 mM		
Spermidine	0 - 1.8 mM	0.1 mM	c-Luc-a	0.3 mM or no spermidine also works for some mRNAs
amino acids	0 - 100 µM	60 µM	c-Luc-a	
DTT	0 - 4 mM	0 mM	c-Luc-a	Should be optimised for each RNA template
tRNA	0 - 150 µg/ml	100 µg/ml	Luc	
Temperature	18 - 37°C	25 °C	c-Luc-a	

Example 3: Comparison between method of the invention and that described in Scott *et al.*, (1979) *Biochemistry*, 18(8): 1588-1594.

Drosophila embryo extracts were prepared in parallel either according to the method described in Scott *et al.* (1979) or to the method of the invention. These extracts were assayed for translation using either the conditions described in Scott *et al.* (1979) or the conditions described above.

Briefly, the Scott protovol consisted in dechorionating the emryos in a solution of 50% ethanol/50% chlorox for 1 m in under agitation, washing the embryos 5 times in PBS after dechorionation using a table-top centrifuge at top speed to obtain the embryo pellet and homogenising the embryo pellet in 10mM HEPES pH7.4, 6mM β-mercaptoethanol.

The extracts obtained using this method were used in an *in vitro* translation reaction using either the conditions described in Example 2.1, those described by Scott *et al* (1979) or those described by Scott with an increased time of translation. The RNA used

for translation encoded the firefly luciferase and contained either a cap (c), a poly(A) (a), or both (c-a). The results (Figures 1 and 2) indicate that by using our extract in our *in vitro* translation conditions a high degree if translation and synergism was obtained. When the Scott extract was used under Scott's *in vitro* translation conditions,. no synergism was obtained and a very low level of translation was observed. Using combination of both extracts with either ours or Scott's *in vitro* translation conditions, low levels of translation with various degrees of synergy were obtained. Any apparent synergy in this figure exhibited by the Scott extract using our conditions is believed only to be because the very low level of translation seen gives unreliably low levels of luciferase activity. Accordingly, the (c), (a) and (c-a) figures cannot be compared. These data indicate that the combination of our extract (Example 1) with our *in vitro* translation conditions (Example 2.1) make possible a high degree of translation and synergism.

Comparative results of translation levels and levels of synergy found between the 5' cap and the 3' poly(A) tail of the mRNA template are shown in Figures 1 and 2.

Translation levels using the Scott method were very low. Translation levels using the extract and method of the invention were high and the level of synergy noted was also high (12x and 15x). Figure 2 shows the results of Figure 1 on an expanded scale.

Example 4: Preparation of cell-free extracts from HeLa cells

HeLa cells were maintained at exponential growth in suspension cultures at 37° C in Jocklik's Medium supplemented with 5% newborn bovine serum. Approximately 204 litres of cells at densities of 3-6 x 10^5 cells/ml were harvested by centrifugation at 700 g for 15 minutes and washed three times with phosphate buffered saline (PBS) at 4° C.

Pelleted cells were resuspended in 1 volume of ice-cold hypotonic buffer containing Hepes 10 mM, pH 7.6; KOAc 10 mM; $Mg(Oac)_2$ 0.5 mM; and Dithiothreitol 5 mM. Reduced amounts of DTT are not recommended and addition of protease inhibitors is optional.

After 2-10 minutes on ice, cells were broken with 10-30 strokes of a tight-fitting Dounce homogeniser (pestle type B).

Cell lysates were centrifuged at 14,000 g for 5 minutes (a longer centrifugation is not recommended) at 4° C. Supernatant fluid was divided into aliquots frozen in liquid nitrogen and stored at -80° C. Addition of glycerol is not necessary.

Example 5: In vitro translation assay

Incubation mixtures contain:

• untreated extract	40% of the final volume
• Hepes buffer, pH 7.6	16 mM (+4 mM of the lysate)
• KOAc	50 mM (+4 mM of the lysate)
• $Mg(Oac)_2$	2.5 mM (+0.2 mM of the lysate)
• amino acid mixture	100 µM
• spermidine	0.1 mM
• ATP	0.8 mM
• GTP	0.1 mM
• Creatine phosphate	20 µM
• Creatine kinase	50 ng/µl
• Calf liver tRNA	100 ng/µl
• RNA template	1 ng/µl

Reaction mixtures, typically at a final volume of 12.5µl were incubated at 37° C for 90 minutes.

In order to obtain these optimal values for the translation assay, a range of concentrations of the main components were tested with a capped and polyadenylated mRNA expressing the bacterial enzyme Chloramphenicol Acetyltransferase (CAT). Translational efficiency is judged by the amount of the CAT protein (ng) measured by a colorimetric enzyme immunoassay (CAT ELISA, Böhringer Mannheim).

Two sets of transcripts coding for the reporter enzymes CAT (Preiss. T. & Hentze. M. W. 1998 Nature 392:516-520) and firefly luciferase. Luc, (Iizuka. N. et al. 1994 Mol. Cell. Biol. 14:7322-7330) were tested in the translation assay and the functional half life of each transcript has been determined to be as follows:

- 5' capped (Cap, m7Gppp) 40 minutes

- 5' capped and polyadenylated (pA, 98 adenines) 55 minutes
- uncapped <10 minutes
- uncapped and polyadenylated <10 minutes

Figure 8 shows a northern blot analysis of the transcripts during the translation assay. Bars represent radioactive intensity of each messenger measured by a phosphoimager. Graph (A) shows the four messengers at different times, from 0 to 150 min. Graph (B) shows the Cap and the Cap-pA transcripts from 0 to 90 min.

The efficiency of translation of capped mRNAs having a polyA tail is thus remarkably higher than the one lacking it. Using several batches of CAT transcripts, it has been observed that differences between capped and capped-polyadenylated messengers are in a range of 15 to 60 fold. Uncapped mRNA are poorly translated and are also rapidly degraded.

In order to study the PolyA effect independently from the presence of the Cap, the stability of the Luc transcripts was enhanced using the cap structure analog G(5')ppp(5')A (Acap).

Figure 9 shows the time course of translation of the Luc messengers. Efficiency of translation is represented by light emission measured by a luminometer. Graph (B) shows the data represented in graph (A) with the y axis maximum at 20000 luminescence instead of 550000.

The functional half life of each transcript has been determined to be :

- Cap: 40 minutes
- Cap and pA: 40 minutes
- Acap: 25 minutes
- Acap and pA: 60 minutes
- uncapped: 12 minutes
- uncapped and pA: 20 minutes

The synergism of the polyA and the Cap structures is shown by the 40 fold difference of translation of the Cap-pA mRNA with respect to the sum of the translation of the two mRNAs Cap and Acap-pA.

References

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